Protein Targeting to the Inner Nuclear Membrane: Possible roles of the ER gene *ICE2* and Components of the Spindle Pole Body

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with research distinction* in Molecular Genetics in the undergrad colleges of The Ohio State University

by

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June 2008

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ABSTRACT

The nucleus is surrounded by a nuclear envelope (NE) consisting of two layers, the outer nuclear membrane and the inner nuclear membrane (INM). The ER is contiguous with the outer membrane and the two layers are connected at the nuclear pores. The INM is an essential structure, consisting of a number of proteins that are involved with the structural organization of the nucleus. Individual components of the NE play important roles in cell biology and human health. Defects in the INM protein, laminA, result in nuclear laminopathies known to cause at least sixty-four independent diseases, including muscular dystrophies and progeria. Despite the importance of the NE, how it assembles and how the structure is maintained is not well understood. It was discovered that the deletion of the *ICE2* gene in *Saccharomyces cerevisiae* caused the mislocalization of a reporter protein, Trm1-II, that normally localizes on the INM (Murthi and Hopper, 2005). Nine proteins that exhibit localization similar to Trm1 were selected for testing to determine if Ice2p plays a general role in targeting proteins to the INM. Through homologous recombination, the *ICE2* gene was deleted in strains containing chromosomally encoded candidate proteins fused in frame to GFP. If Ice2p functions in targeting of the candidate proteins to the INM, then the deletion of *ICE2* will result in mislocalization of the proteins. So far, six of the nine candidate proteins have been assayed for localization in *ICE2* deletion strains. Four have been observed to exhibit wild type localization and Asi1-GFP has been found to mislocalize in strains containing the *ICE2* deletion. Also, prior research has shown that spindle pole body (SPB) components may also function in appropriate distribution of Trm1-GFP. In yeast, the SPB is the microtubule organizing center, the functional equivalent of the centrosome.

The SPB organizes the mitotic spindle and cytoplasmic microtubules throughout the cell cycle and is anchored in the nuclear envelope. To determine if loss of function of SPB components affects targeting of other INM proteins, I chose the same nine INM proteins that are being used in the *ICE2* deletion study. As the SPB components are essential, temperature sensitive mutants were used. Candidate genes were PCR amplified and supplied with a GFP tag for direct visualization as well as a galactose inducible promoter. The cells will be grown in the presence of galactose to induce transcription and then incubated at non permissive temperature (37°C). The cells will then be observed by fluorescence microscopy. In the SPB project, still in progress, the candidate genes are currently being amplified and supplied with an in frame GFP and a promoter. Some candidates were studied for the localization of Trm1p.

CHAPTER 1: INTRODUCTION

One of the features that separate the eukaryotic cell (Fig. 1.1) from the prokaryotic cell is the presence of a membrane-bound nucleus. The membrane, known as the nuclear envelope, consists of two lipid bilayers joined together by the nuclear pore complexes (NPC). The two layers consist of the outer nuclear membrane (ONM) which is contiguous with the endoplasmic reticulum (ER) and the inner nuclear membrane (INM). The 20-40 nm gap in between the two layers is known as the perinuclear space (Fig. 1.1).

The INM contains several transmembrane or integral proteins and peripherally associated proteins. Proper targeting of these proteins to the INM is very important, as evidenced by families of diseases which are associated with the inappropriate targeting of proteins to the INM. These diseases are collectively known as laminopathies and include certain types of muscular dystrophies like Emery-Dreifuss Muscular Dystrophy and Limb Girdle Muscular Dystrophy as well as certain types of progeria like Hutchinson-Gilford Progeria syndrome (Gruenbaum *et al*., 2003). Despite the importance of proper INM location, the mechanism of how these proteins are properly targeted and maintained at the nuclear periphery is not yet fully understood.

The budding yeast, *Saccharomyces cerevisiae,* has been a powerful model organism for geneticists for decades and it is a good model organism to study the nucleus and the nuclear membrane. Unlike higher eukaryotes, yeast undergoes closed mitosis where the nucleus does not completely break down and reassemble. Instead, the NE extends and pinches off to form two nuclei during mitosis. Due to the difference between

yeast and higher eukaryotes, the *S. cerevisiae* nucleus offers the opportunity to study nuclear membrane organization, while higher eukaryotes prove to be a more challenging model in which to study the nucleus as a result of the nuclear dynamics of open mitosis that renders such analyses very difficult. Despite the differences between yeast and higher eukaryotes, there is evidence that both share very similar mechanisms for nuclear membrane organization. For example, nuclear lamins, which are found in higher eukaryotes but not yeast, localized to the nuclear periphery when expressed in yeast, suggesting some possible conservation in mechanism (Smith and Blobel, 1994). Additionally, yeast is also a well-defined eukaryotic model with highly-developed genetic tools, collections, and a sequenced genome that is also easy to use and readily available. Yeast's ability to propagate with a haploid genome also makes it very easy manipulate for deletions and phenotypic studies.

Previously, two genetic screens were performed in order to identify gene products important for the wt localization of Trm1-II-GFP; one used the yeast deletion collection containing individual deletions of the majority of the ~4000 unessential genes of *S. cerevisiae* and the other screened the temperature sensitive collection of ~300 essential genes, both using the protein Trm1-II-GFP as a reporter. *TRM1* is a non-essential gene encoding two isoforms of a tRNA methyltransferase. Starting at the first AUG, Trm1-I localizes at the mitochondria, whereas Trm1-II, which starts being transcribed at the second AUG, localizes at the INM (Rose *et al*, 1995), both of which catalyze the N^2, N^2-dimethylguanosine base modification in tRNA (Ellis *et al*., 1986, Rose *et al*, 1992). Trm1-II tagged with GFP peripherally associates with the INM in an even distribution similarly to WT Trm1-II detected with an antibody raised against the protein (Rose *et al*,

1992), proving that a GFP signal does not affect Trm1-II's localization and allows for live cell microscopy rendering Trm1-II-GFP as a useful reporter. It was successfully used to identify gene products that are involved in protein targeting to the INM in the two previous genetic screens.

In the genetic screen of the ~4000 non-essential genes in *S. cerevisiae*, one of the interactions uncovered was the deletion of the *ICE2* gene that caused Trm1-II-GFP to mislocalize from the INM and become nucleoplasmic (Fig. 1.2; Murthi and Hopper, 2005). *ICE2* encodes for a type-III transmembrane protein, 491 amino acids in length which localizes to the cortical and perinuclear ER. Deletion of *ICE2* causes defects in the morphology of the ER tubular network indicating that Ice2 contributes to the forming and maintaining the cortical ER network in budding yeast (Estrada de Martin *et al*., 2004). The current model is that Ice2 either regulates tethers to which INM proteins are targeted or is a tether itself.

The second genetic screen of the essential genes used a temperature sensitive collection of ~300 genes (provided by C. Boone) to assay for essential gene products that are involved in protein targeting to the INM (Harchar and Hopper, unpublished). It was found that temperature sensitive mutations of central plaque components of the spindle pole body (SPB) caused Trm1-II-GFP to mislocalize into a spot on the nuclear rim (Fig. 1.2; Harchar and Hopper, unpublished). In *Saccharomyces cerevisiae,* the spindle pole body (Fig. 1.3) is the microtubule organizing center (MTOC) and it is the functional equivalent of the centrosome. It is anchored in the nuclear envelope and organizes the spindle and cytoplasmic microtubules throughout the cell cycle and is essential for successful mitosis. The SPB consists of the outer plaque, which faces the cytoplasm, the

inner plaque, which faces into the nucleoplasm, and the central plaque which helps anchor the SPB to the NE. It is important to note that only the temperature sensitive mutations of components of the central plaque (*spb42-10, spb110-220*), not components of the inner or outer plaque, caused the mislocalization of Trm-1-II.

While Ice2 and the SPB central plaque components, were identified as playing a role in protein targeting, it is not known whether these gene products act specifically on Trm1-II or whether they play a more general role in protein targeting to the INM. In other words, we wanted to ask whether proteins that also seem to localize to the nuclear periphery are affected by *ice2Δ* or temperature sensitive SPB components. To address this question, nine proteins, Asi1, Sir2, Brr6, Gtt3, Sac3, Esc1, Sir3, Mlp1, and Heh2 were chosen to study because they localize to the INM similar to Trm1-II. Each gene was fused, in frame, with GFP to allow for direct live cell visualization. If these proteins mislocalize in strains containing the *ICE2* deletion or the temperature sensitive SPB mutations, then *ICE2* and the SPB plays a general role in protein targeting to the INM. However, if these proteins do not mislocalize, then this would suggest that the process of nuclear targeting is specific to specific proteins or that other major general targeting pathways exist.

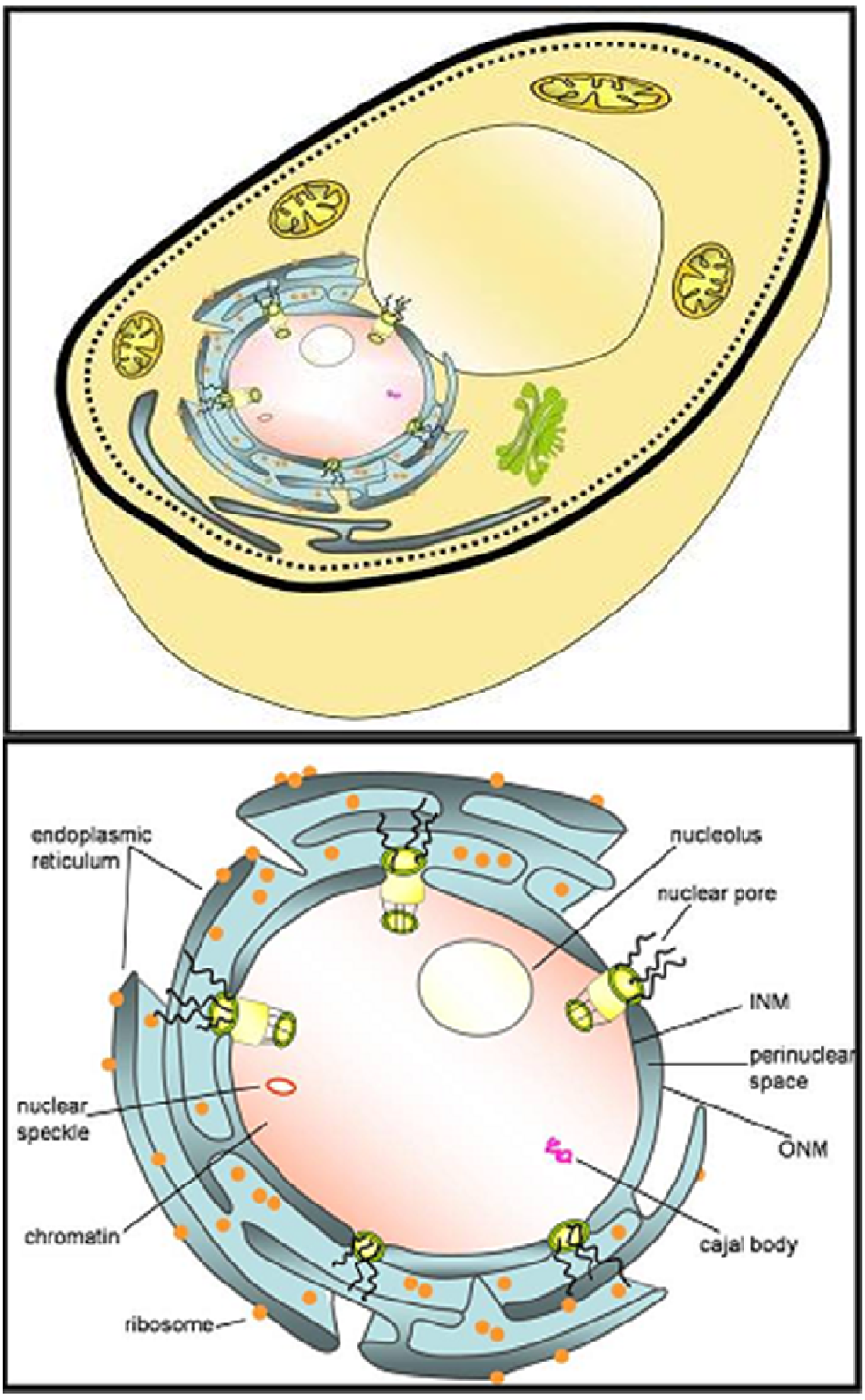

Figure 1.1: Schematic representation of a yeast cell and nucleus.

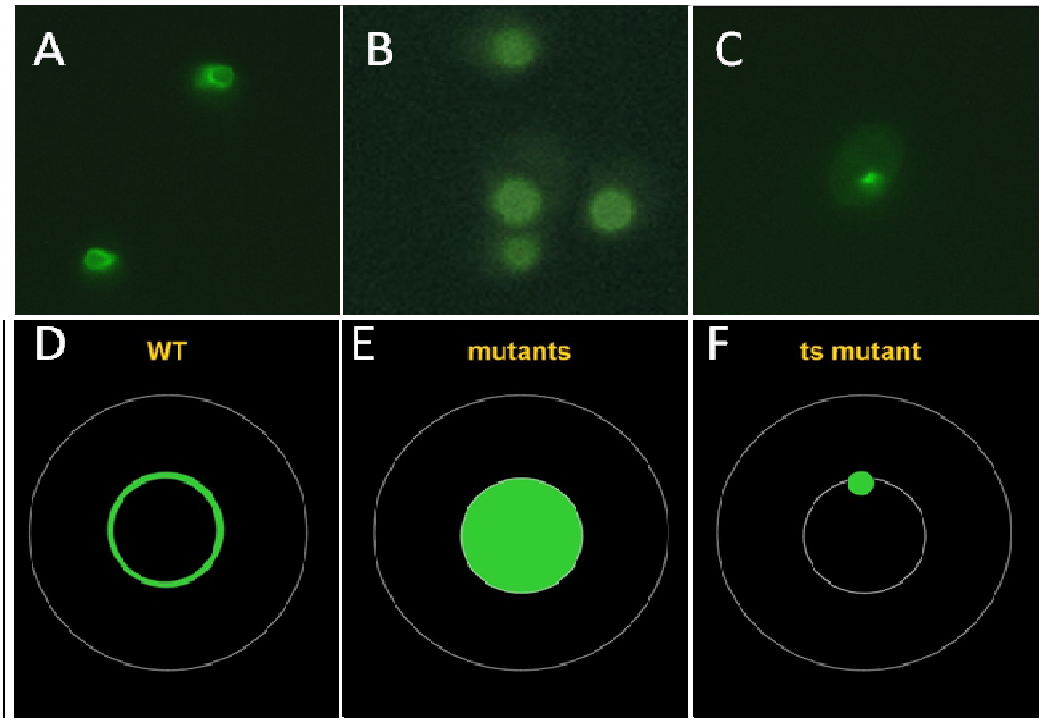

Figure 1.2: Trm1-II-GFP localization in WT (A and D), *ice24* (B and E) and temperature sensitive (C and F) strains. Microscopy pictures from Murthi and Hopper, 2005, Harchar, unpublished.

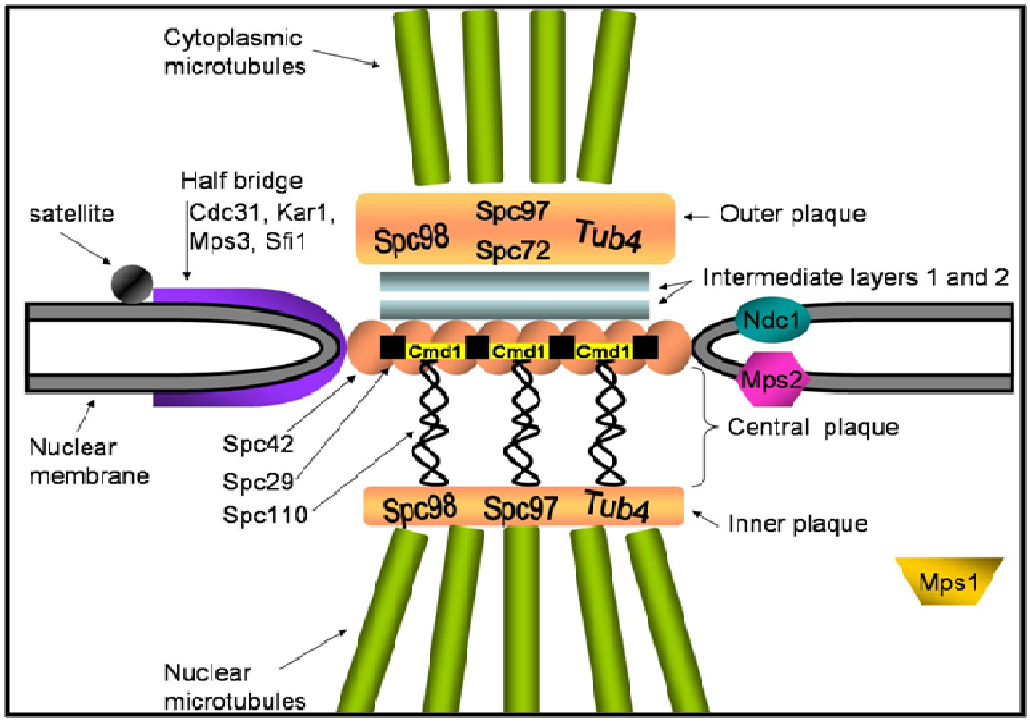

Figure 1.3: Schematic representation of the yeast spindle pole body and the main components (Diaz, 2007).

CHAPTER 2: MATERIALS AND METHODS

Strains and Media

Strains used for study can be found in Table 2.1. Strains were either grown on yeast extract peptone dextrose media (YEPD), YEPD+ clonNat (100mg/L; Werner BioReagents), or synthetic defined media lacking uracil. For galactose induction studies, cultures were grown overnight in synthetic media lacking uracil and glucose but containing raffinose. Then the GAL-promoter was induced by the addition of of 1/10 volume of 20% galactose.

Strain Construction

All deletions were accomplished by lithium acetate transformation (Schiestl and Gietz, 1989) with minor modifications. Briefly, 1.5mL of freshly saturated culture were pelleted and resuspended in 100 μL OSB buffer (0.1M lithium acetate, 0.05M DTT, 40% polyethelene glycol, 0.5 mg/mL of single-stranded salmon sperm DNA). Then 10 μL of the plasmid DNA or PCR product was added to each pellet. The pellets were then incubated at 45°C for 15-30 min. For drug selectable markers, cells were then grown in 3mL YEPD for 3-12 h. The cultures were then spun down in 1.5 mL eppendorf tubes and plated on selective media. For nutritional markers, cells were plated directly onto drop-out media.

All oligonucleotide sequences used in this study are listed in Table 2.2. To generate the *ice2::nat*^{R} knock-out cassette, to replace the endogenous *ICE2* gene, JAW001 and JAW002 were used to PCR amplify nat^{R}-MX4 template (pAG25, Goldstein

and McCusker, 1999). The basic strategy was to use the 50 bp regions homologous to *ICE2* on JAW001 and JAW002 to target the nat^R gene to replace the endogenous *ICE2* gene (Fig 2.1). The 20 bp on the 3' end of JAW001 and JAW002 were complementary to the template allowing PCR amplification (Fig. 2.1)

Genomic Preparation of Transformation Candidates

After streaking for single colonies, candidates were grown to saturation in 1.5 mL of YEPD. Cultures were pelleted and cell walls were removed through digestion with zymolyase. DNA was purified by phenol/chloroform extraction followed by ethanol precipitation of the DNA. DNA was then dissolved in water and used as a template for PCR. To confirm correct integration of the *ice2::nat*^{R} cassette, genomic DNA was PCR amplified using either JAW003 and JAW006 or JAW004 and JAW005, and either JAW006 and RLH025A or JAW005 and RLH025C. The strategy was to confirm the presence of the *ice2::nat*^{R} cassette by hybridizing to the middle of the *nat*^{R} gene and to the genome outside of the recombination region. To confirm the absence of the *ICE2* gene, amplification of *ICE2* was attempted by hybridizing to the middle of the *ICE2* gene and the genome outside of the recombination region.

Plasmids

Plasmids used in this study are listed in Table 2.3.

Plasmids constructed in this study were made in collaboration with Greetchen Diaz. The following genes were PCR were amplified using Hi-Fi Taq (Invitrogen); *SAC3* (GDM001, GDM007), *GTT3* (GDM003, GDM004), *ASI1* (GDM023, GDM024), *ESC1*

(GDM025, GDM026), *HEH2* (GDM027, GDM028), *MLP1* (GDM029, GDM030), *SIR3* (GDM033, GDM034), and *SIR2* (Athula57, Athula58). After gel extraction, PCR products were ligated into pGEM-T vector (Promega) per protocol and then transformed into chemical competent *E. coli*. Plasmids extracted from ampicillin-resistant colonies were digested using the following restriction enzyme combinations: SmaI (*SAC3*), SmaI and HindIII (*GTT3*), XmaI (*ASI1, ESC1, HEH2, MLP1, SIR3*), and EcoRI and HindIII (*SIR2*). Digestion products were separated using agarose gel electrophoresis and desired gene containing bands were extracted. Gel extracted DNA was then ligated into pGp54a that was previously digested with one of the following combinations of restriction enzymes: SmaI (*SAC3*), SmaI and HindIII (*GTT3*), XmaI (*ASI1, ESC1, HEH2, MLP1, SIR3*), and EcoRI and HindIII (*SIR2*). Ligation products were then transformed into chemical competent *E. coli* and plasmids were extracted from ampicillin-resistant colonies. Correct insertion of genes into plasmids was confirmed through PCR amplification using a GAL-promoter specific primer (Athula44) and the downstream primer specific to that gene.

Fluorescence Microscopy

Fluorescence microscopy and image capture was done with Nikon90i equipped with a Cool-SNAP HQ2 digital camera and MetaMorph software (Molecular Devices, Sunnyvale, CA). Images were assembled using Microsoft Powerpoint 2007.

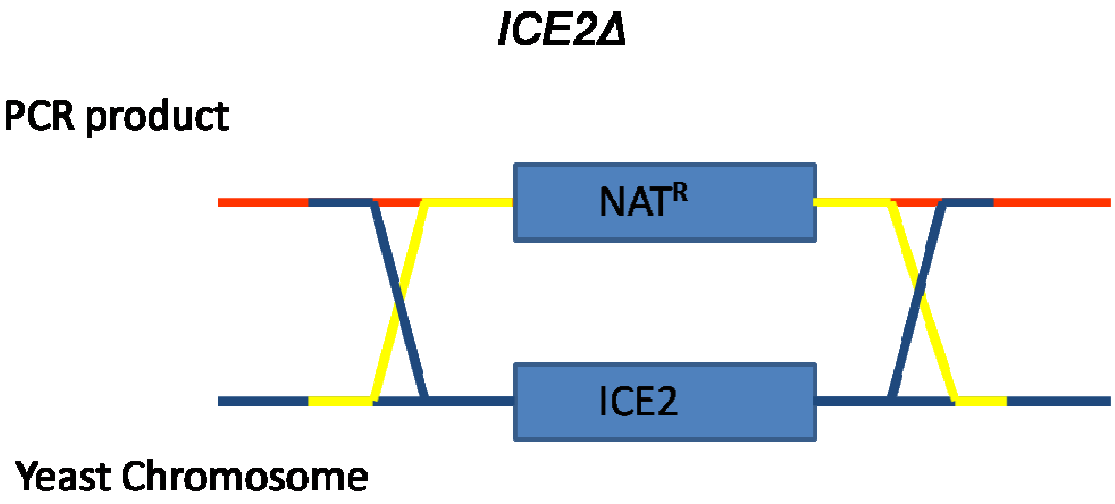

Fig. 2.1: nat^{R} replaces *ICE2* through homologous recombination

Table 2.1: Yeast Strains

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	Invitrogen
Asi1-GFP	*MATa YMR119W-GFP-HIS3MX6-his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	Huh et al., 2003; Invitrogen
Brr6-GFP	*MATa YGL247W-GFP-HIS3MX6-his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	Huh et al., 2003; Invitrogen
Esc1-GFP	*MATa YMR219W-GFP-HIS3MX6-his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	Huh et al., 2003; Invitrogen
Gtt3-GFP	*MATa YEL017W-GFP-HIS3MX6-his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	Huh et al., 2003; Invitrogen
Heh2-GFP	*MATa YDR458C-GFP-HIS3MX6-his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	Huh et al., 2003; Invitrogen
Mlp1-GFP	*MATa YKR095W-GFP-HIS3MX6-his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	Huh et al., 2003; Invitrogen
Sac3-GFP	*MATa YDR159W-GFP-HIS3MX6-his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	Huh et al., 2003; Invitrogen
Sir2-GFP	*MATa YDL042C-GFP-HIS3MX6-his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	Huh et al., 2003; Invitrogen
Sir3-GFP	*MATa YLR442C-GFP-HIS3MX6-his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	Huh et al., 2003; Invitrogen
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	Invitrogen
Asi1-GFP *ice2Δ*	*MATa YMR119W-GFP-HIS3MX6 ice2::natMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	This study
Brr6-GFP *ice2Δ*	*MATa YGL247W-GFP-HIS3MX6 ice2::natMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	This study
Esc1-GFP *ice2Δ*	*MATa YMR219W-GFP- HIS3MX6 ice2::natMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	This study
Gtt3-GFP *ice2Δ*	*MATa YEL017W-GFP- HIS3MX6 ice2::natMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	This study
Heh2-GFP *ice2Δ*	*MATa YDR458C-GFP-HIS3MX6 ice2::natMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	This study
Mlp1-GFP *ice2Δ*	*MATa YKR095W-GFP-HIS3MX6 ice2::natMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	This study
Sac3-GFP *ice2Δ*	*MATa YDR159W-GFP-HIS3MX6 ice2::natMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	This study
Sir2-GFP *ice2Δ*	*MATa YDL042C-GFP-HIS3MX6 ice2::natMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	This study
Sir3-GFP *ice2Δ*	*MATa YLR442C-GFP-HIS3MX6 ice2::natMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study

	TRP1 LYS2	
spc42-10	*MATa spc42-10-kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	C.Boone, University of Toronto
spc110-220	*MATa spc110-220-kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TRP1 LYS2*	C. Boone, University of Toronto

Table 2.2: Oligonucleotides used in this study

Name	**Sequence (5' – 3')**	**Source**
JAW001	CTATTAGAGAGGTGCTGTTTGTGGCCGATCACGCTAAAGATTAGGCAACGCCAGGTCGACGGATCCCCGG	This study, PSU Molecular Core
JAW002	CGCGTATTTGGCAAAGCTGGTAATGCGGCTCTTTTGACAATGCATCAGCTCGTGGATCTGATATCATCGA	This study, PSU Molecular Core
JAW003	CGTGGCAATTCGTGCTTACCAAGTCCACAC	This study, Invitrogen
JAW004	GTGTGGACTTGGTAAGCACGAATTGCCACG	This study, Invitrogen
JAW005	CCTCAGCCCATCGCGTAATTGC	This study, Invitrogen
JAW006	GTCAGTTGGTATTTCACCTTCC	This study, Invitrogen
RLH025A	CCG GCG GAT GGG GTT CAC	Hurto, R., unpublished, PSU Molecular Core and Invitrogen

RLH025C	GTGTCGGTGGTGAAGGACCCATCC	Hurto, R., unpublished, PSU Molecular Core and Invitrogen
GDM001	CCCGGGATGAACACATCATTTGGCTCA	Greetchen Diaz, Invitrogen
GDM003	CCCGGGATGCCGACCAAGTCAACTTTTAGT	Greetchen Diaz, Invitrogen
GDM004	AAGCTTTAATACGTATAGAGTGAGGAG	Greetchen Diaz, Invitrogen
GDM007	CCCGGGATCATTATTTACTTTCTTCTTGAC	Greetchen Diaz, Invitrogen
GDM023	CCCGGGATGAACTCTTCCACTTCT	Greetchen Diaz, Invitrogen
GDM024	CCCGGGTTTACTATCTGAAACAGG	Greetchen Diaz, Invitrogen
GDM025	CCCGGGATGTCAAAAAAAAAAGAAACCT	Greetchen Diaz, Invitrogen
GDM026	CCCGGGTTTGTCAACACTTGGATG	Greetchen Diaz, Invitrogen

GDM027	CCCGGGATGGATCACAGAAACCTT	Greetchen Diaz, Invitrogen
GDM028	CCCGGGTTCTTTCCATTCCCAACA	Greetchen Diaz, Invitrogen
GDM029	CCCGGGATGTCGGATCATGATACT	Greetchen Diaz, Invitrogen
GDM030	CCCGGGTATACTGTTCTCGTCATT	Greetchen Diaz, Invitrogen
GDM033	CCCGGGATGGCTAAAACATTGAAA	Greetchen Diaz, Invitrogen
GDM034	CCCGGGAATGCAGTCCATATTTTT	Greetchen Diaz, Invitrogen
Athula44	GGCAGTAACCTGGCCCCACAAACCTTC	Athula Murthi, Invitrogen
Athula57	GAATTCATGACCATCCCACATATGAAATACGCCG	Athula Murthi, Invitrogen
Athula58	AAGCTTGAGGGTTTTGGATGTTCATCTGATG	Athula Murthi, Invitrogen

CHAPTER 3: RESULTS

Effects of the *ICE2* Deletion

Since *ICE2* is a non-essential gene, it is able to be removed or disrupted without drastically affecting the viability of the cell. In this study, *ICE2* was deleted through homologous recombination with a PCR product containing Nat^R, a drug resistance marker, flanked by 50 bp of sequences directly up and down stream of the *ICE2* coding region. The yeast strains used for this study contained the genomically encoded reporter protein with C-terminal GFP tags. These proteins were chosen through the *Saccharomyces* Genome Database (www.yeastgenome.org) through a search for proteins that had localization at the nuclear periphery. Localization was then viewed through the GFP Database at UCSF. In this manner, candidate proteins were narrowed to nine proteins that localized to the nuclear periphery and appeared to localize to the INM in a similar manner to Trm1-II-GFP. Out of the nine proteins chosen, *ICE2* has been deleted in six of the strains: those containing Esc1-GFP, Gtt3-GFP, Sac3-GFP, Mlp1-GFP, Sir3-GFP and Asi1-GFP.

Localization of Esc1, Gtt3, Sac3, and Mlp1 is not affected by the deletion of ICE2

Esc1 is a protein that localizes to the inner nuclear periphery and is responsible for silencing chromatin and tethering telomeres and is essential for the partitioning and anchoring of plasmids by another nuclear periphery protein, Sir4 (Andrulis *et al*., 2002). Unlike the even distribution of Trm1-II-GFP, Esc1-GFP has a patchy distribution along the INM likely due to its association with telomeres (Andrulis *et al*., 2002). When observed with fluorescence microscopy, it appeared that there was no change in the cellular distribution of Esc1-GFP within the strain containing the deletion (Fig 3.1 C, D)

when compared to the wild type strain (Fig. 3.1 A, B). Thus, it does not appear that Ice2 influences the localization of Esc1.

Little is known about Gtt3. It is predicted to play a role in glutathione metabolism by a computational analysis of large-scale protein-protein interaction data (Samanta and Liang, 2003). Gtt3-GFP localizes to the nuclear periphery with a smooth distribution similar to Trm1-II-GFP. Again, the localization of Gtt3-GFP in the *ICE2* deletion strain (Fig. 3.1 E, F) looked very similar to the localization of Gtt3-GFP in the wild type strain (Fig. 3.1 G, H). Therefore, it can be concluded that the *ICE2* deletion does not affect the localization of Gtt3p.

Sac3 is an mRNA export factor that was originally identified in a screen for suppressors of actin mutations (Novick *et al*., 1989) and has been shown to be required for normal mitotic progression and for spindle morphology (Bauer and Kölling,. 1996). Sac3 was also shown to localize to the inner nuclear rim and associate with the nuclear pore complexes which results in an uneven INM distribution. Sac3-GFP localized as expected in the wild type strain (Fig. 3.2 A, B). Deletion of *ICE2* did not alter the localization of Sac3-GFP (Fig. 3.2 C, D).

Mlp1 is known to localize to the inner nuclear periphery, specifically connecting the nuclear pore complex with the nuclear interior (Strambio-de-Castillia *et al*., 1999). Its uneven distribution across the INM could possibly be explained by Mlp1's association with the nuclear pore complexes. It is involved in telomere length control (Hediger *et al*., 2002) and in nuclear retention of unspliced mRNAs (Galy *et al*, 2004). It was found that the localization of Mlp1-GFP in the wild type strain (Fig. 3.2 E, F) and the localization of

Mlp1-GFP in the strain containing the *ICE2* deletion (Fig. 3.2 G, H) were very similar, again suggesting that Ice2 does not play a role in the localization of Mlp1-GFP.

Transformants with a confirmed deletion of *ICE2* were unable to be obtained in strains containing Sir2-GFP, Heh2-GFP, and Brr6-GFP Since the candidate proteins were fused with GFP in the chromosome, each protein was expressed under normal wild type conditions. Depending on how a protein is expressed normally, it is possible to get high amounts of protein or low amounts of protein depending on the function. A confirmed deletion of *ICE2* in the strain containing Sir3-GFP was obtained; however, expression level was too low for us to assay for changes in localization using fluorescence microscopy.

Asi1 is mislocalized in the presence of the ICE2 *deletion*

Unlike many of the other proteins that were studied, Asi1 is an integral INM protein, meaning that at least one transmembrane domain is present in the protein. Asi1, a glycoprotein, is predicted to have five transmembrane domains as well as a zinc-binding RING motif (Boban *et al*., 2006). In complex with Asi2 and Asi3, it sequesters full-length unprocessed forms of transcription factors, Stp1 and Stp2, that escape cytoplasmic regulation, maintaining the repression of amino acid permease genes under noninducing conditions (Zargari *et al*., 2007). Asi1-GFP forms a ring, similar to the appearance of Trm1-II-GFP. Asi1-GFP localized as expected in the wild type strain, but in the strain containing the *ICE2* deletion, Asi1-GFP seemed to mislocalize to the ER, suggesting that Ice2 does play a role in targeting Asi1 to the INM under normal conditions.

Effects of the temperature sensitive SPB central plaque component mutants on protein localization

Over expression of the candidate proteins does not allow for adequate observation of protein localization.

The same nine proteins that were studied in the *ICE2* deletion assay were chosen to be studied in the SPB component assay. The central plaque proteins of the SPB are essential, meaning that the cell's viability is dependent on the proper function of these proteins. Because these genes are essential, they cannot be deleted like *ICE2* can. Instead, it is necessary to use temperature sensitive versions of these genes. At non-permissive temperature (37°C), the temperature sensitive gene products are rendered unable to function. Thus it is possible to observe the effect of non-functional SPB central plaque components on the localization of proteins that reside at the INM. In order to achieve this, the candidate genes were PCR amplified and then ligated into a plasmid containing GFP with which to tag the proteins for visualization. This plasmid also contains a galactose inducible promoter which allows for controlled expression of the GFP constructs. If the GFP protein was synthesized constitutively, it would be impossible to see how the protein localizes when the temperature is shifted to non-permissive temperature due to the GFP protein synthesized before the temperature shift, which would be already residing at the INM. With the galactose inducible promoter, we are able to synthesize the GFP protein before shifting to non-permissive temperature, allowing us to assay how the temperature sensitive mutations are affecting the localization of the candidate proteins.

Plasmid construction was completed. The galactose promoter was induced for 30-60 min, however, it was found that the overexpression of the candidate proteins rendered it difficult to assay for any changes between the wild type strains and the temperature sensitive strains under permissive and non-permissive temperature (Fig. 3.3, 3.4). It is not an effect of the temperature, nor of the temperature sensitive mutations themselves because we see that the effect is present both at permissive and at non-permissive temperature as well as in both wild type strains and temperature sensitive strains. Therefore, at this time, we are unable to answer the question of if the components of the central plaque of the SPB have a global effect on proteins that target to the INM.

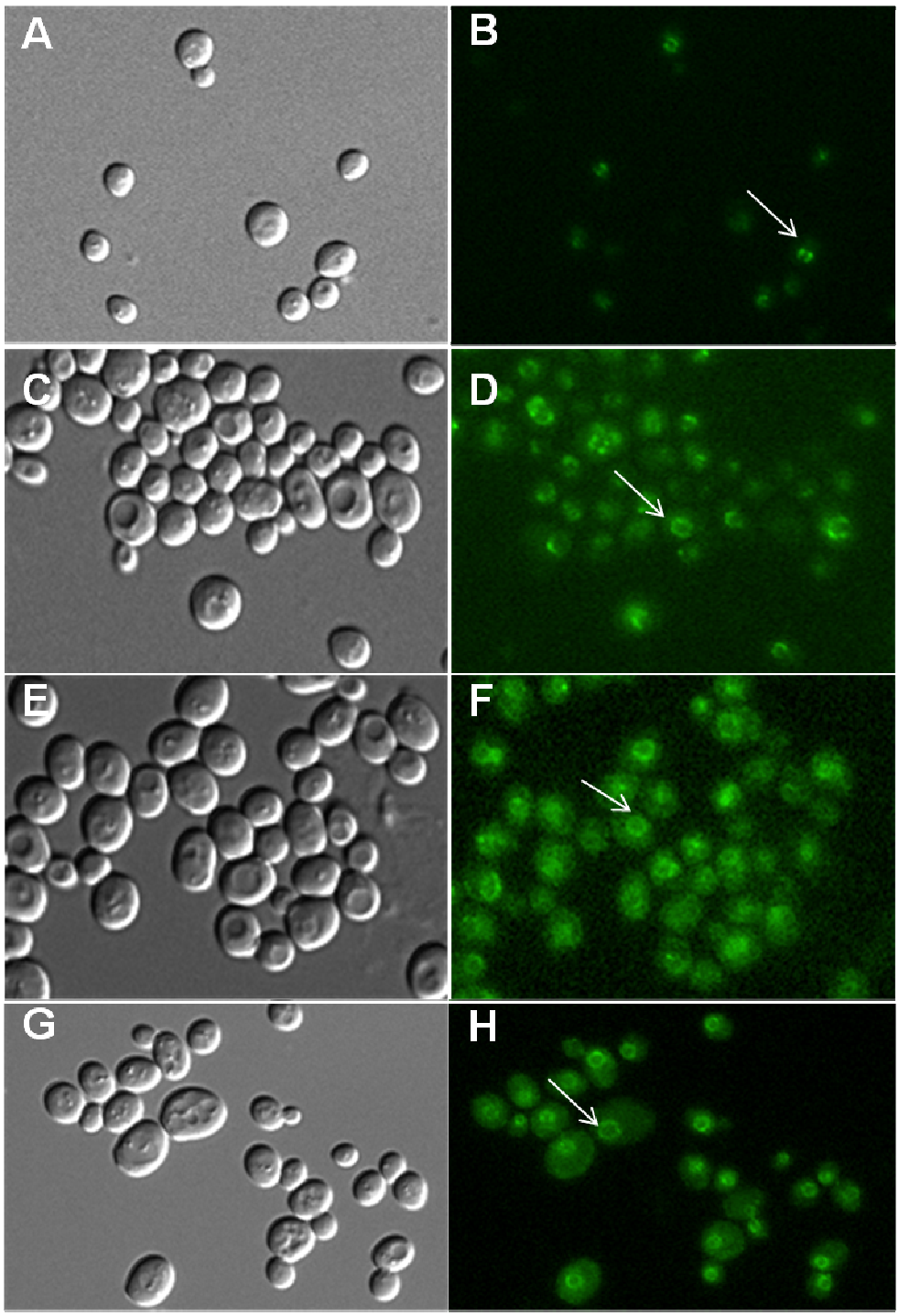

Figure 3.1: DIC and fluorescence microscopy of ESC1-GFP strains WT (A-B), ESC1-GFP ice2Δ (C-D), GTT3-GFP WT (E-F) and GTT3-GFP ice2Δ (G-H).

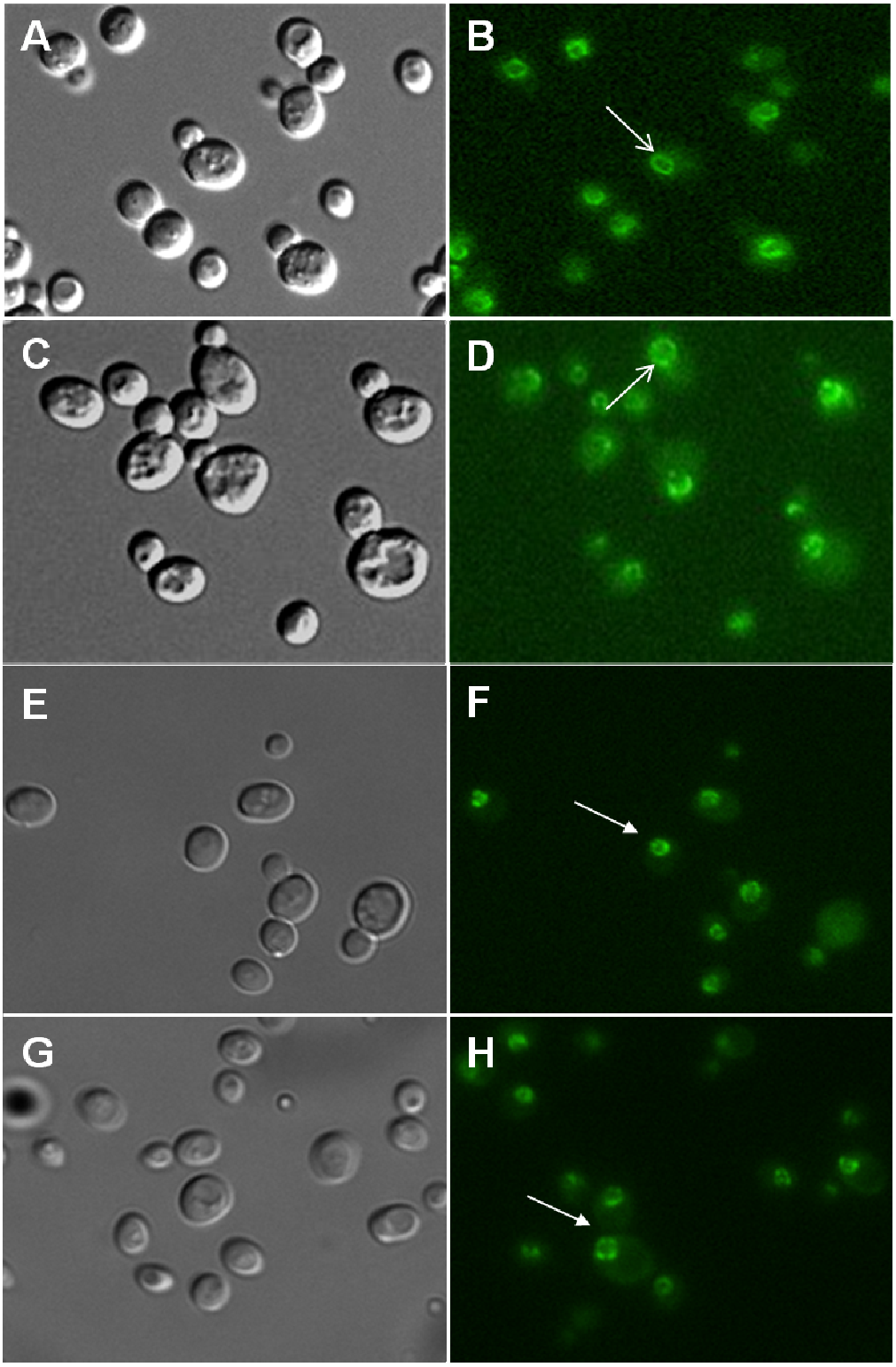

Figure 3.2: DIC and fluorescence microscopy of SAC3-GFP strains WT (A-B), SAC3-GFP ice2Δ (C-D), MLP1-GFP WT (E-F) and MLP1-GFP ice2Δ (G-H).

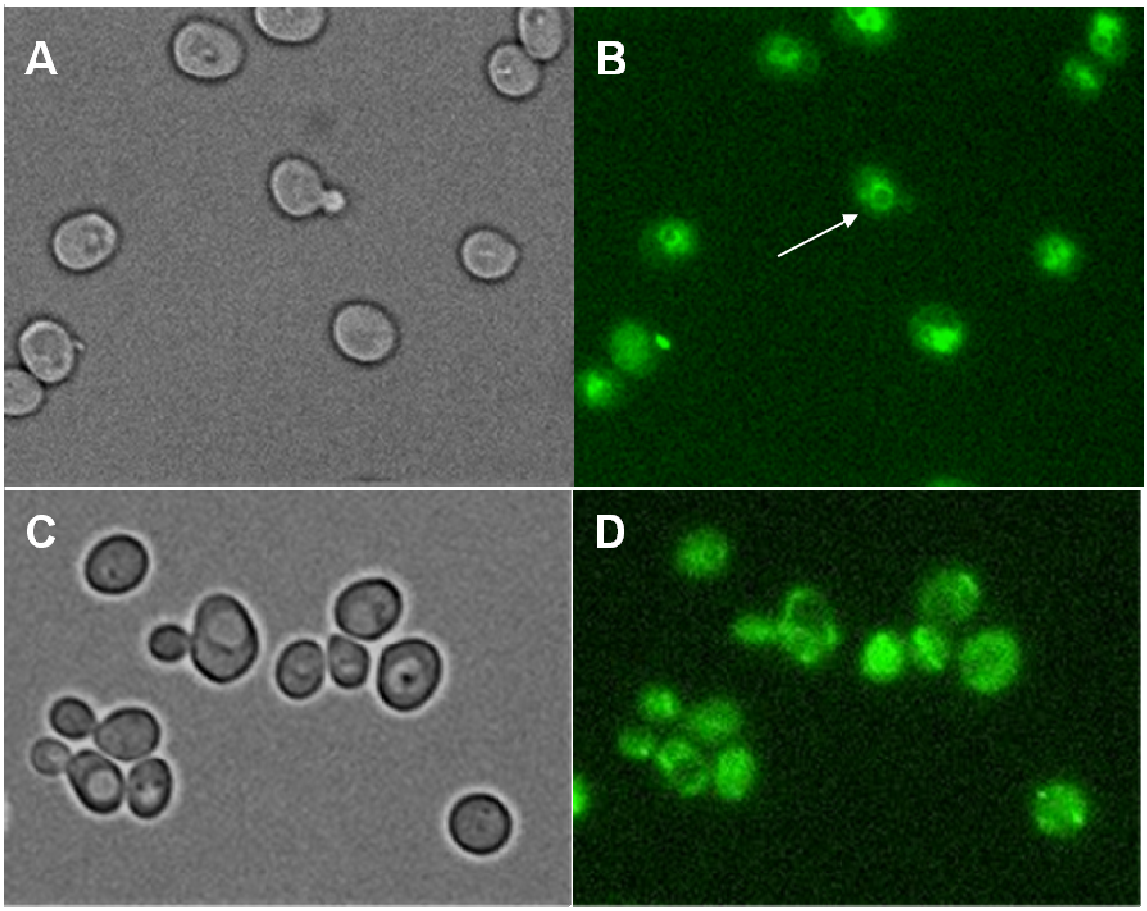

Figure 3.3: DIC and fluorescence microscopy ASI1-GFP WT (A-B) and ASI1-GFP ice2Δ (C-D).

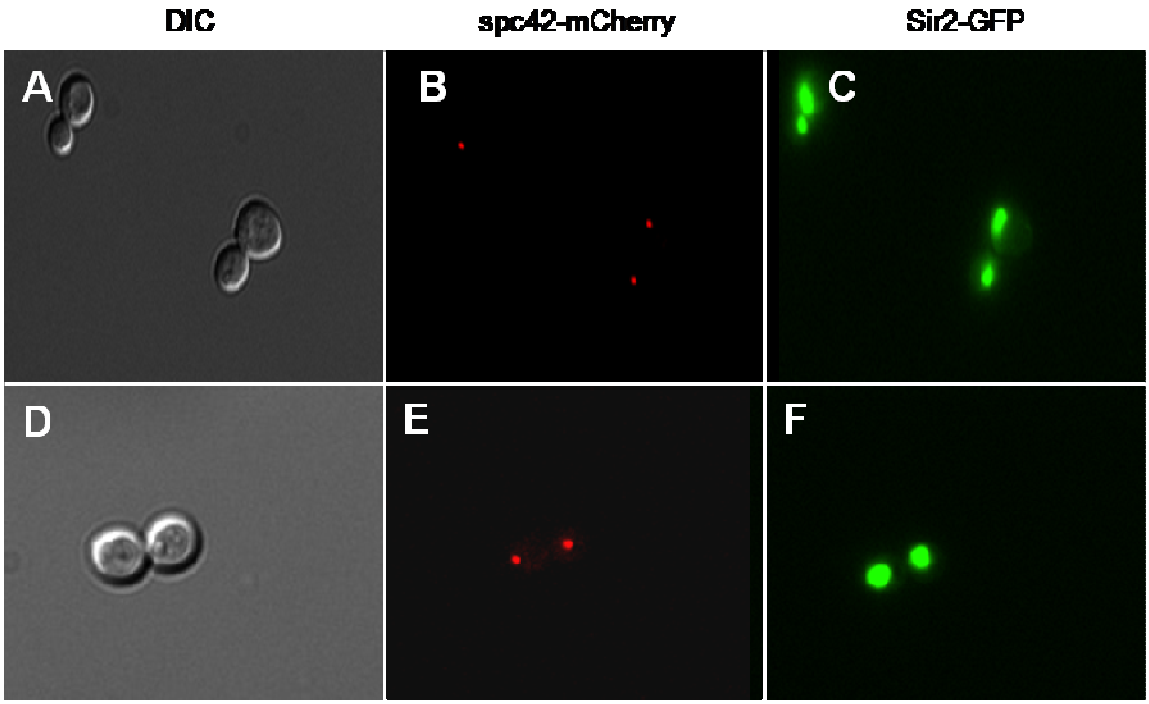

Figure 3.4: DIC and fluorescence microscopy of WT: spc42-mCherry; Sir2-GFP at 23°C (A-C) and at 37°C (D-F). Strains were induced with galactose for 30-60 min.

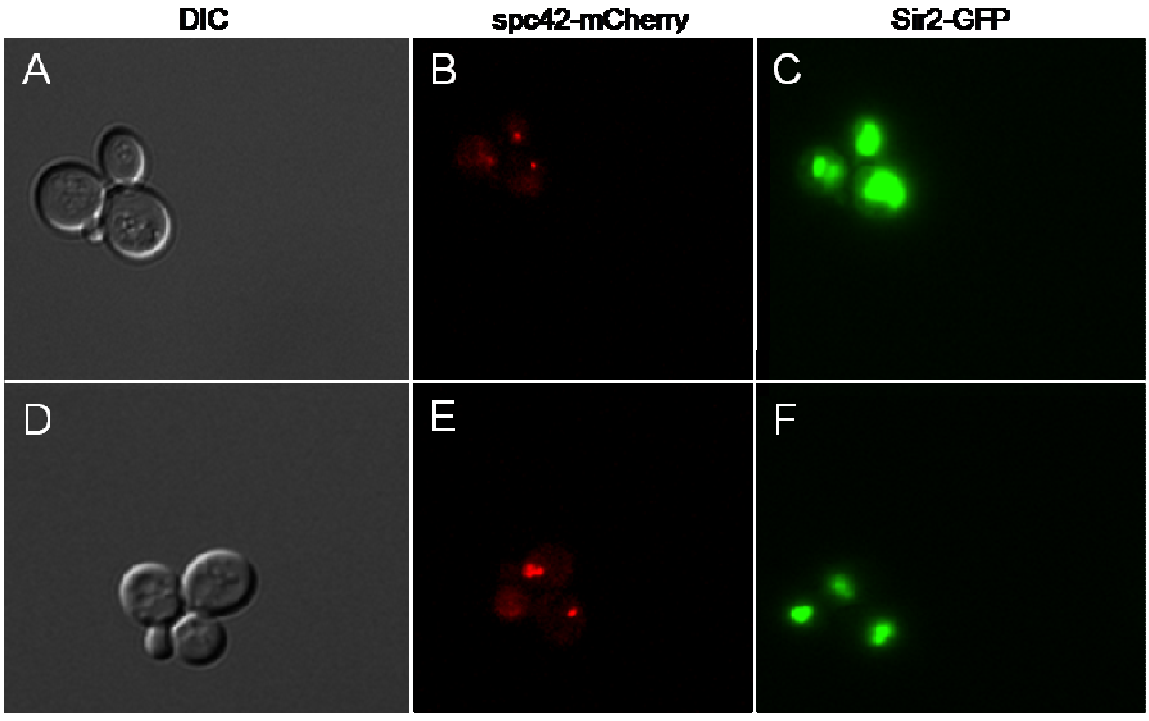

Figure 3.5 DIC and fluorescence microscopy of *spc110-220*: spc42-mCherry; Sir2-GFP at 23°C (A-C) and at 37°C (D-F). Strains were induced with galactose for 30-60 min.

CHAPTER 4: DISCUSSION

A major factor in the proper function of the eukaryotic cell is the nuclear membrane. The maintenance of its structure and its biogenesis are of great interest given the discoveries revealing roles for the INM in cellular functions such as gene regulation. Proper targeting of proteins to the INM is important for these processes. Many human diseases such as muscular dystrophies and progeria can be traced to improper protein targeting to the INM. Despite the critical role that INM protein targeting plays, the exact mechanisms that enable these proteins to arrive and maintain at the proper location are not well-defined. The two previous genetic screens of the non-essential and the essential genes of *S. cerevisiae* revealed the cortical ER protein Ice2 and components of the central plaque of the SPB to be important in the proper targeting of a reporter protein, Trm1-II-GFP. Though these gene products function in targeting Trm1-II-GFP to the INM, it was not known whether they specifically affected Trm1-II-GFP or whether they played a more global role in protein targeting to the INM. The goal of this project was to answer this question through observing the localization of nine proteins that targeted to the INM similarly to Trm1-II-GFP when *ICE2* was deleted or at non-permissive temperature in strains carrying temperature sensitive versions of the genes that encoded SPB central plaque proteins. The results of this work found that the *ICE2* deletion did not affect the localization of Esc1-GFP, Gtt3-GFP, Sac3-GFP, and Mlp1-GFP. However, Asi1-GFP was mislocalized in the absence of Ice2 to what appears to be the ER. Future experiments with Asi1-GFP, *ice2Δ*, and an ER marker can confirm this finding.

These results show that protein targeting defects of the *ICE2* deletion is not specific to Trm1-II-GFP, indicating that Ice2p appears to regulate a general mechanism

for protein targeting. However, the fact that it does not affect the localization of all the proteins indicates that at least one additional mechanism functions to properly target proteins to the INM. Future work would include continuing to assay for localization defects in the presence of the *ICE2* deletion in other proteins that normally reside at the INM to better define how global of a role Ice2 plays in the localization of INM proteins. Though the previous studies focused on proteins that associate peripherally to the INM, the mislocalization of Asi1-GFP, an integral membrane protein, suggests that the focus should be shifted to include other integral membrane proteins as well. For example, Asi2 and Asi3 would be good candidates to test their localization in strains containing the *ICE2* deletion. The results indicate that, at the very least, Trm1-II-GFP and Asi1-GFP are involved in the same targeting mechanism that is regulated by Ice2.

Though both Trm1-II-GFP and Asi1-II-GFP mislocalized in the presence of the *ICE2* deletion, they mislocalized in very different ways. Trm1-II-GFP becomes nucleoplasmic, while Asi1-GFP does not appear to enter the nucleus at all, instead, remaining in what appears to be the ER. This may be due to Trm1-II-GFP being a peripheral INM protein while Asi1-GFP is an integral INM protein. Since Asi1 is an integral protein, it is conceivable that it might mislocalize to the ER since one function of the ER is to fold and sort proteins meant to localize at membranes. Since Ice2 localizes to the ER, it was hypothesized that either Ice2 regulates tethers to which INM proteins are targeted or that it may be a tether itself. These results support the current model for Ice2 function in INM targeting where Ice2 may help sort an integral membrane protein to the INM that functions as a tether for Trm1-II-GFP. If Asi1-GFP is the only integral membrane protein that is affected by the deletion of *ICE2*, it could be possible that Asi1

itself is the tether to which Trm1-II tethers. This question can be answered by observing the localization of Trm1-II-GFP in an *asi1Δ* strain. It is also possible that Ice2 itself is the tether to which these proteins localize, but this seems unlikely since the deletion of *ICE2* affects an integral membrane protein which can act as tethers but are not tethered themselves.

Interestingly, in a genome-wide study, the *ICE2* deletion was uncovered as being synthetically lethal with the deletion of *KAR3* (Tong *et al*., 2004). Kar3p is required for nuclear fusion during mating (Rose, 1996), participates in spindle formation, and localizes to the SPB (Page *et al*, 1994). The synthetic lethality of *ice2Δkar3Δ* may also suggest that *ICE2* plays a role in proper chromosome segregation. The data suggests that Ice2 indirectly affects the organization of the INM and indicates a relationship between Ice2 and the SPB that goes deeper than the fact that they both affect the localization of Trm1-II-GFP, but at this point, is not well-characterized.

For the SPB assay, it is necessary to find a novel way to control the expression of proteins tagged with GFP while avoiding overexpression. The expression of the candidate proteins must be controlled carefully and should be able to be turned off and on at will since it is necessary to see how the new protein is localizing when the temperature is shifted to non-permissive temperature. If the protein is expressed constitutively, we would be unable to see any changes in localization between permissive and non-permissive temperature due to some of the candidate protein already localized at the INM by the time of the temperature shift. When a working protocol is developed, it will be interesting to see how the SPB central plaque components affect the localization of these proteins. It will also be interesting to see just how global the effect of the *ICE2* deletion

is on the localization of INM proteins, though the results have indicated that at least one other major targeting pathway exists which has yet to be uncovered.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Anita Hopper for her guidance as my advisor and mentor as I worked on this project and I would also like to thank the rest of my thesis committee, Dr. Susan Cole and Dr. Juan Alfonso for their time and patience as well. I would especially like to acknowledge Greetchen Diaz with whom I collaborated on certain parts of this project for her advice and support as well as the rest of the Hopper lab, especially for the time and advice they gave me while I was working on my project and as I was writing this thesis. I'm especially grateful for the editing. I would also like acknowledge C. Boone for the use of the ts strains.

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